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APPLICATION FOR A UNITED STATES PATENT

In the name of

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For

SCREENING ASSAY BASED ON THE FORKHEAD TRANSCRIPTION FACTOR-DEPENDENT *SOD-3* PROMOTER

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SCREENING ASSAY BASED ON THE FORKHEAD TRANSCRIPTION FACTOR-DEPENDENT *SOD-3* PROMOTER

FIELD OF THE INVENTION

The present invention relates to a process for the screening and identification of compounds modulating directly or indirectly the FOXO forkhead transcription factor activity ("FOXO activity"), transgenic *C. elegans* suitable for the said process, the compounds identified by the said process which modulate the FOXO activity, the use of such compounds for the treatment of disorders and the preparation of pharmaceuticals.

BACKGROUND OF THE INVENTION

In abundant food, *C. elegans* develops through four distinct larval stages (L1-L4) to the adulthood. However, when conditions become less favorable, the development is arrested and an alternative third-stage larvae is formed which is specialized for dispersal and long-term survival, termed *dauer. Dauer* larvae don't feed, are long-lived and resistant to stress. Morphologically, they can be distinguished from adults because they are thinner, darker, and have a constricted pharynx. The changes in morphology correlate with dramatic alterations in the expression pattern of genes in *dauers* and adults. (Riddle, 1988; Riddle and Albert, 1997)

In the past, temperature-sensitive strains have been identified that are *dauer*-constitutive; e.g., at the restrictive temperature of 25°C these strains form *dauers* even in the presence of food (Gems, 1998). It turns out that many of these strains, termed *daf* strains, have acquired mutations in genes involved in the nematode insulin/IGF-1 signaling pathway. Studies of the phenotypes have allowed certain *daf* genes to be ordered into a genetic pathway consisting of *DAF-2*/IR, *age-1*/PI-3 Kinase, *pdk-1*, *akt-1, akt-2*, and the FOXO transcription factor *DAF-16* (Gottlieb and Ruvkun, 1994; Riddle, 1977; Riddle et al, 1981, Kaestner et al., 2000).

It has been shown by Northern blotting and RT-PCR that the expression of the *sod-3* gene is regulated by mutations in the *DAF-2*/insulin receptor pathway (Honda & Honda, 1999). Inactivation of the *DAF-2* function in certain mutant strains results in a

strong up-regulation of the *sod-3* expression. Honda & Honda suggested that DAF-16 is the transcription factor activating the *sod-3* gene and that DAF-16 is inhibited by the *DAF-2*/IR pathway.

Furthermore, a consensus sequence binding to the transcription factor DAF-16 has been identified and this sequence was shown to be present in the *sod-3* upstream regulatory region (Furuyama et al., 2000). This binding motif fused to a minimal promoter was sufficient for insulin-regulated expression in mammalian tissue culture systems.

Since the *DAF-2*/insulin receptor pathway and its components are very well conserved in man, it was proposed to use the *dauer* phenotype to identify modulators of the insulin/IGF-1 signaling in man (WO 98/51351 A1). However, the assay systems according to the prior art require long incubation times until the developmental program of the *dauer* larvae has been completed (usually 3-5 days). Such a long time period may result in the degradation of the assay components. Moreover, the impermeable cuticula structure of *dauers* together with the reduced food-intake might be a setback for compound uptake into the worm.

Therefore, it was the underlying problem of instant invention to provide a process for the identification of compounds that modulate the *DAF-2*/IR pathway, which does not depend on *C. elegans* dauer larvae and overcomes the above-mentioned disadvantages. The process of the invention (i.e., the assay system of the invention) relies on a data read-out that is directly linked to the *DAF-2*/IR pathway, and which is not influenced by the progress of developmental stages of the organism under investigation, preferably mammalian and nematode cells, particularly nematode cells, e.g., *C. elegans*. Furthermore, the assay should provide quantitative data read-out after a short incubation time, preferably within about 8-12 hours, in the presence of the compound(s) to be investigated. Depending on the reporter used in the assay, a quantitative data read-out is obtainable in contrast to the prior art assay systems.

DETAILED DESCRIPTION OF THE INVENTION

It was found by the instant invention that the use of a nucleic acid molecule having the biological activity of an *sod-3* gene promoter element surprisingly is of great

advantage for the identification of genes or compounds that modulate the activity of the *DAF-2*/IR pathway, e.g., the *sod-3* promoter as deposited at the Deutsche Sammlung für Zellkulturen und Mikroorganismen, Mascheroder Weg 1b, D-38124 Braunschweig, Germany, DSMZ No. 14912 (the 1098 bp fragment after endonuclease digestion with HindIII and BamHI) on April 4, 2002, especially the *sod-3* promoter according to Seq. ID No. 1. This regulatory DNA fragment contains the binding site for the FOXO DAF-16 that is functionally linked to the *DAF-2*/IR pathway via *akt-1*. In spite of current knowledge of the daf2/IR signalling pathway, a suitable responsive promoter element to monitor signalling activity for *C. elegans* has not been known in the art. When the *sod-3* promoter is fused to reporter genes, rapid quantification of the DAF-2/IR activity can be achieved. The instant invention provides thereby the great advantage that quantification of the DAF-2/IR activity is independent of strain background or developmental stages of the *C. elegans*, which – according to the prior art - had to be synchronized.

Accordingly, one embodiment of the present invention is an isolated nucleic acid molecule comprising a promoter exhibiting the biological activity of the sod-3 promoter. Preferably, the nucleic acid sequence of the invention is selected from the group consisting of: (a) a nucleic acid sequence comprising the nucleic acid sequence of SEQ ID NO. 1; (b) a nucleic acid sequence that has 80%, 90%, 95% or greater sequence identity to the nucleic acid sequence of (a) having *sod-3* promoter activity; (c) a fragment of the nucleic acid sequence of (a) or (b) having *sod-3* promoter activity; and (d) a derivative of the nucleic acid sequence of (a), (b) or (c) having sod-3 promoter activity, preferably a DNA or RNA molecule, more preferably having a 80%, 90%, 95%, or greater sequence identity to SEQ ID No. 1; and (e) a nucleic acid sequence that hybridizes, preferably under stringent conditions, to SEQ ID NO:1. A still more preferred embodiment of the nucleic acid molecule according to the invention comprises a promoter exhibiting the biological activity of the sod-3 promoter in nematodes, preferably in *C. elegans*.

According to instant invention, a promoter exhibiting the biological activity of the sod-3 promoter means any promoter, which is responsive to forkhead transcription factors, preferably, the FOXO forkhead transcription factors (hereinafter "FOXO's"),

particularly, DAF-16. Such promoters are, e.g., FOXO1a, FOXO3a or FOXO4 responsive promoters" (Kaestner et al, 2000).

According to the instant invention the term "fragment" means any parts of the nucleic acid molecules of the invention, which are long enough in order to exhibit the biological activity of the sod-3 promoter.

According to the instant invention the term "derivative" means that the sequence may differ from the sequences of the nucleic acid molecules of the invention at one or more positions, exhibiting a high degree of homology to these sequences. Hereby, "homology" means a sequence identity of at least 50%, in particular an identity of at least 60%, preferably of more than 80% and still more preferably a sequence identity of more than 90%. The deviations with respect to the above-described nucleic acid molecule might have been caused by deletion, substitution, insertion or recombination. Moreover, homology means a functional and/or structural equivalence.

The invention further encompasses nucleic acid sequences that hybridize to nucleic acid sequence of SEQ ID NO:1. A nucleic acid molecule is "hybridizable" to another nucleic acid molecule, such as a cDNA, genomic DNA, or RNA, when a single stranded form of the nucleic acid molecule can anneal to another nucleic acid molecule under the appropriate conditions of temperature and solution ionic strength. The conditions of temperature and ionic strength determine the "stringency" of the hybridization. Low stringency hybridization conditions correspond to a T_m of 55° C (*e.g.*, 5x sodium chloride/sodium citrate (SSC), 0.1% SDS, 0.25% milk, and no formamide; or 30% formamide, 5x SSC, 0.5% SDS). Moderate stringency hybridization conditions correspond to a higher T_m, (*e.g.*, 40% formamide, with 5x or 6x SSC). High stringency hybridization conditions correspond to the highest T_m, (*e.g.*, 50% formamide, 5x or 6x SSC). Hybridization requires that the two nucleic acids contain complementary sequences, although depending on the stringency of the hybridization, mismatches between bases are possible. The appropriate stringency for hybridizing nucleic acids depends on the length of the nucleic acids and the degree of complementation, variables well known in the art. The greater the degree of similarity or homology between two nucleotide sequences, the greater the

value of T_m for hybrids of nucleic acids having those sequences. The relative stability (corresponding to higher T_m) of nucleic acid hybridizations decreases in the following order: RNA:RNA, DNA:RNA, DNA:DNA. For hybrids of greater than 100 nucleotides in length, equations for calculating T_m have been derived. For hybridization with shorter nucleic acids, *i.e.*, oligonucleotides, the position of mismatches becomes more important, and the length of the oligonucleotide determines its specificity.

In a particular embodiment of the present invention, a hybridizable nucleic acid molecule of the invention hybridizes under stringent conditions to the nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO:1, a complement thereof, or a fragment thereof. The term "hybridizes under stringent conditions" is describes conditions for hybridization and washing under which nucleotide sequences at least 55%, 60%, 65%, 70% and preferably 75% or more complementary to each other typically remain hybridized. Such stringent conditions are known to those skilled in the art and can be found in "Current Protocols in Molecular Biology", John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. A preferred example of stringent hybridization conditions are hybridization in 6X SSC at about 45° C, followed by one or more washes in 0.2X SSC, 0.1% SDS at 50-65°C.

Another embodiments of instant invention are isolated nucleic acid molecules comprising the said nucleic acid sequence according to the invention exhibiting *sod-3* promoter activity and a nucleic acid sequence conferring the activity of a reporter gene ("fusion molecule"); vectors comprising the nucleic acid molecules according to the invention, which may further be optionally linked to regulatory elements which ensure the transcription and the synthesis of a translatable RNA of a reporter gene in eukaryotic cells or transgenic host cells transformed with the nucleic acid molecule or the vector of instant invention.

Still another embodiment of the invention is the transgenic host or host cell transfected with the nucleic acid molecule or the vector of the invention, which is preferably of nematode origin and the method for their preparation comprising the steps of generating a transgenic host cell, preferably of nematode origin, by use of

the nucleic acid molecule or the vector of the invention.

Yet another embodiment of the present invention is a process for the identification of modulators of the *DAF-2*/IR pathway, AKT pathway and/or of kinases phosphorylating one or more FOXO's (i.e. the "Screening Assay" according to the invention) comprising the said transgenic cell or transgenic organism, preferably a nematode (e.g., C.elegans), according to the invention.

A preferred embodiment of the invention is a process for the identification of modulators of the *DAF-2*/IR pathway, AKT pathway, of kinases phosphorylating, phosphatases dephosphorylating, and/or other activities (e.g., enzymes) altering the molecular composition, stability (i.e., half-life), subcellular location, or activity of one or more FOXO's comprising

(a) bringing transgenic *C. elegans*, preferably L1 larvae, into contact with one or more compounds to be tested for the ability to modulate the *DAF-2*/IR pathway, AKT pathway, of kinases phosphorylating, phosphatases dephosphorylating, and/or other activities (e.g., enzymes) altering the molecular composition, stability (i.e., half-life), subcellular location, or activity of one or more FOXO's under suitable conditions, said transgenic *C. elegans*, preferably L1 larvae, comprising the nucleic acid molecule of the invention fused to a reporter gene or the vector of the invention comprising said fusion molecule;

(b) measuring the reporter gene activity in the presence of one or more compounds to be tested;

(c) measuring the reporter gene activity in the absence of the one or more compounds to be tested, optionally in the presence of one or more suitable reference compounds;

(d) comparing the reporter gene activities of steps (b) and (c); and

(e) selecting the modulating compound(s) of the *DAF-2*/IR pathway, AKT pathway, of kinases phosphorylating, phosphatases dephosphorylating, and/or other activities (e.g., enzymes) altering the molecular composition, stability (i.e., half-life), subcellular location, or activity of one or more FOXO's.

Another embodiment of instant invention is a process for the identification of modulators of the *DAF-2*/IR pathway comprising

(a) bringing a transgenic *C. elegans* L1 larvae into contact with one or more compounds to be tested for the ability to modulate the *DAF-2*/IR pathway under stressful condition, said L1 larvae comprising the nucleic acid molecule of the invention fused to a reporter gene or the vector of the invention comprising said fusion molecule;

(b) measuring the amount of L1 larvae, which enters into *dauer* larvae state under the condition of step (a) in the absence and in the presence of one or more compounds to be tested, optionally in the presence of one or more suitable reference compounds;

(c) comparing the amounts of L1 larvae, which entered into *dauer* larvae state according to step (b); and

(d) selecting the modulating compound(s) of the DAF-2/IR pathway.

According to the instant invention the term "modulator" means any chemical molecule or genetic element, which has an inhibitory, activatory or regulatory effect on the *DAF-2*/IR pathway, AKT pathway, of kinases phosphorylating, phosphatases dephosphorylating, and/or other activities (e.g., enzymes) altering the molecular composition, stability (i.e., half-life), subcellular location, or activity of one or more FOXO's.

According to the instant invention the term "suitable reference compound" means a vanadate salt, e.g., sodium orthovanadate, monoperoxo(picolinato)oxovanadate(V), or potassium bisperoxo(1,10-phenanthroline)oxovanadate (V).

According to the instant invention the term "suitable condition" means any cultivation condition suitable for *C. elegans* known by the person skilled in the art (e.g., see Sulston & Hodgkin, 1980).

According to the instant invention the term "stressful condition" means any cultivation condition suitable for *C. elegans* known by the person skilled in the art, which differ from suitable conditions in that they are essentially sub-optimal without killing the

worm, preferably, conditions, which are known to induce Dauer larvae formation (e.g., see Sulston & Hodgkin, 1980).

The Screening Assay of the invention exhibits great advantages in comparison to conventional assays (e.g., assays using exit from *dauer* larvae state) with respect to speed of the performance of the assay, feasibility of quantification, and avoidance of side effects, e.g., developmental side effects.

Quantifiable reporter genes suitable to practise the assay systems according to instant invention may encode for proteins that can be detected due to their enzymatic or fluorescent properties such as luciferase, ß-galactosidase, ß-lactamase, secreted alkaline phosphatase, green fluorescent protein, coral reef fluorescent proteins, or other reporters known to the skilled artisan (e.g., Hill et al, 2001). Reporter activity might be measured in lysates of the organisms or *in-situ* in the living cell or animal.

Activation of the reporter reveals in the identification of inhibitors of the *DAF-2*/IR or AKT pathway, while a down-regulation of the reporter activity is indicative for activators of the said pathway.The reporter might be used in wild-type *C. elegans* or in combination with certain strains that might contain mutations in genes associated with, for example, the *dauer* pathway, preferably *daf-2* mutant strains.

The identified compounds, which inhibit the signaling of the *DAF-2*/IR pathway components are promising candidates as therapeutic agents in the field of oncology and cardiac hypertrophy, while activators of the said pathway are promising candidates as therapeutic agents in the treatment of diabetes, brain/heart ischemia, or neurodegenerative diseases.

EXAMPLES

The following examples are not to be understood as limiting the invention but shall merely illustrate the inventive concept:

Material and methods

Genomic DNA was prepared from wild type *C. elegans* (N2) using proteinase K and phenol extraction as described previously (Sulston and Hodgkin, 1980).

The *C. elegans* vectors pPD49.26 and pPD95.75 were used according to Fire et al. (Methods in Cell Biology, Vol. 48, Chapter 19 (C. Mello and A. Fire), Academic Press).

Example 1

Isolation of the *sod-3* promoter

To isolate the regulatory sequences of the *sod-3* gene, 1266 bp upstream of the start codon were amplified from wild type *C. elegans* (N2, Bristol, Caenorhabditis Genetics Center, 250 Biological Science Center, University of Minnesota, 1445 Gortner Avenue, St. Paul, MN 55108-1095, USA) genomic DNA by polymerase chain reaction with the upstream primer sod-5U (Seq. ID No. 2) and the downstream primer sod-3U (Seq. ID No. 3), adding a 3´ BamHI restriction site to the PCR product. The oligonucleotide primers used were as follows:

forward sod-5U: 5´-agttttaaagattttattcatagtcc-3´ (Seq ID No. 2);
reverse sod-3D: 5´-ggatcctttattcactgaaaattagaagatt-3´ (Seq ID No. 3).

Subsequently, the identity of the resulting 1266 bp PCR product was confirmed by sequencing. The GFP expression vector was assembled by cloning into the pPD49.26 backbone a) the 1098 bp BamHI and HindIII fragment of the *sod-3* promoter and b) and a PCR fragment of GFP amplified from pPD95.75 containing flanking restriction sites for NheI and KpnI.

The resulting in a *C. elegans* expression vector containing the *sod-3*::GFP fusion was termed pMGC2-24

Example 2

Transgenic *C. elegans*

daf-2(e1368) animals and transgenic animals were obtained according to a standard procedure (Mello and Fire, 1995). In contrast to the method of Mello and Fire, the plasmid pMGC2-24 was injected together with the injection marker *ttx-3*::GFP into

the gonads of the said animals. Three independent lines were isolated by isolation of GFP-positive animals.

Example 3

Specific read-out for the *DAF-2*/Insulin receptor pathway

The regulation of the *sod-3* promoter was demonstrated by comparing the expression of *sod-3*::GFP in *daf-2(e1368)* animals at different temperatures. The *daf-2*(e1368) strain contains a temperature-sensitive mutation in the ligand-binding domain of *DAF-2*/IR resulting in an inactivation of DAF-2 at 25°C. When L1 larvae were grown up at the permissive temperature of 15°C for 4 days, a weak expression of GFP could be detected in the tail, head, and in the vulva of the adults animals. The overall expression of GFP was quite low. This changed dramatically when L1 larvae were grown up at the restrictive temperature of 25°C with a concomitant inactivation of DAF-2. Under these conditions, the *C. elegans* were arrested as *dauers* and GFP fluorescence was strongly up-regulated in the whole animal. The up-regulation of *sod-3*::GFP was abolished in a *daf-2(e1368)* strain which had an additional deletion in the *daf-16* gene. Likewise, under these experimental conditions, wild-type N2 worms with normal *DAF-2*/IR function kept at 25°C neither formed *dauers* nor did they respond with an increase in *sod-3* expression.

Therefore, the regulation of the *sod-3*::GFP expression correlated with the inactivation of the *DAF-2*/IR pathway in the *daf-2(e1368)* strain at 25°C. The data are in agreement with a model in which the *DAF-2*/IR pathway acts to inhibit the transcription factor *DAF-16* which otherwise activates the transcription of the *sod-3* gene. Therefore, the reporter is activated when the *DAF-2*/IR pathway is switched off and deactivated when the *DAF-2*/IR pathway is switched on.

Example 4

The *sod-3*::GFP reporter is regulated independent of the developmental stage

daf-2(e1368) animals containing the *sod-3*::GFP reporter were kept at 15°C until they finished the development to adults and were then shifted as adults to 25°C (restrictive temperature) to inactivate DAF-2/IR. As seen with *dauers*, also adults exposed to the restrictive temperature expressed much more GFP in comparison to

animals kept at the permissive temperature of 15°C. Densitometric scanning revealed an increase from 2.6±1.7 mean GFP at the permissive temperature to 53.5±14.6 mean GFP at the restrictive temperature. The increase in GFP expression in the adults is in the same order of magnitude as seen with L1 shifted immediately to 25°C to give dauers (mean GFP: 87.8±35.3). This suggests that the regulation of the *sod-3* promoter is independent of the developmental stage of the *C. elegans*, and that up-regulation of the *sod-3* promoter as a consequence of the inactivation of the *daf-2*/IR gene can be induced at any time. Consequently, the *sod-3 C. elegans* strain can be used for screening with adult *C. elegans* thus avoiding potential interference of compounds with nematode development. In addition, incubation times can be shorter since the assay is not dependent on the completion of the developmental program.

SEQ ID No:1 (*sod-3* promoter (HindIII x BamHI fragment of DSMZ plasmid pMGC 2-24). The sequence begins and ends with the restriction sites of HindIII, resp., BamHI:

aagcttaaaaatagcagaatttgcaaaacgagcaggaaagtcatattcgcagaaaaaagtcgttgcaaacattcgttt
ttatatgtttttctttgagaaagcgtggttcatttttgaaagtgaaaaatatttgcttaaaacttccaaatttaaatctgcagtga
ttcagagaggttgagaattattttcaaaaacattcaatgttttcccttggagtgactatgcaaatatgaaaatgttttccaaa
aatatttggatgccctgataaaaagtaggtgaaatttcgcaggggaacatcatattaaaatgttgaatttttagaagaaat
ggaaatgtttgtcggtggtatgctcgaatatttgagatattatatatttactgttaaatccgaaatttttgacaaacggaaaa
aatttgtgtcgaaatactacattttcgataacacaaaggtacttccataacacttataaaaactgtttgactatcttatttcag
gaaaaaaaaatccaagaataaacatttttcagaatttgaactttctaatggctgattaataaaacaaagttatacaacta
ttcaaagcagttgctcaatctggcattttcttgtgttttttttttgaatatttcatcagcaagatgttgataattttgtgttaattctaat
tgttttctacaatttttcaaaccgaaaattgacctttgactttgtttactttgttctcgtgggttaactgttcactgatttctattgctg
ttgatgaggtctttgatcaaatttgtattgtttttatactgcatattgcttcaattctaaatcatctaatatattgtcaaacaacttct
tgttttttttttcattcaaaacttctgcaaaaacgttctcttaacaaaggttcacacaacaactctcctctccatctctttctctca
acaacaatgtgctggccttgcatgtttgccagtgcgggttgtttacgcgttttcaagatttttggtctcctatctaacgtcccg
aaatgcatttttttcctttcatttggtttttttctgttcgagaaaagtgaccgtttgtcaaatcttctaattttcagtgaataaaggat
cc

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The foregoing references, as well as all other references cited herein, are incorporated herein by reference in their entirety.